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Susceptibility of dermatophytic arthrospores to physical and chemical agent.

Antidermatomycotic effect of glutaraldehyde

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Effects of various physical, chemical and biological agents on arthrospores of Trichophyton mentagrophytes and conditions affecting germination of dermatophytic arthrospores were investigated. The efficacy of glutaraldehyde as a practically useful antidermatophytic agent was tested in a simulated laundry condition and its usefulness for disinfecting clothes contaminated with arthrospores and infective hyphae of T. mentagrophytes was confirmed in simulated laundry tests. Although arthrospores of this

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dermatophyte were highly resistant to chilling and freezing, they were extremely susceptible to moderate heat (above 50°C) and desiccation. The high susceptibility of arthrospores to desiccation could be significantly reduced when they were dried in the presence of exogenous proteins. Arthrospores of this fungus were markedly susceptible to glutaraldehyde. Arthrospores of T. mentagrophytes appeared to be significantly more resistant to common antimycotics such as clotrimazole, griseofulvin, miconazole nitrate and nystatin than its hyphal counterpart. Clinical and epidemiological implications of these observations were discussed. Germination of dormant arthrospores occurred only in rich complex media such as Sabouraud dextrose broth or vitamin-free Casamino Acids. However, once activated, arthrospores were able to germinate under wide ranges of pH (5.5 to 8.0, optimal 6.5) and temperature (20 to 39°C, optimal 37°C) in the presence of certain single amino acids or oligopeptides known to be present in the human cutaneous tissues. Dormant arthrospores could be activated by incubation in distilled water at 25°C for 24 h or by brief exposure to sublethal doses of heat (45°C for 10 to 20 min). Approximately 20% of activated arthrospores underwent spontaneous germination at 37°C during an additional 18 h of incubation in distilled water. All monosaccharides, purines, pyrimidines, and nucleosides tested failed to induce germination of T. mentagrophytes arthrospores. Germination rate was affected by the concentration of germination inducers as well as that of arthrospores. The germination process of T. mentagrophytes arthrospores was found to be oxygen dependent and was relatively tolerant to NaCl, clotrimazole, cycloheximide, griseofulvin, and tolnaftate.

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(6) CONTROL OF DERMATOMYCOSES BY PHYSICAL, CHEMICAL AND BIOLOGICAL AGENTS

by

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Dermatophytes produce two types of spores depending on the environments in which they grow. Conidia (micro- and macroconidia) are produced when they are grown in vitro (soil, artificial laboratory media, etc.). When growing in parasitized tissues such as skin, hair and nail, many dermatophytes produce arthrospores by segmentation of hyphae. It has been suspected that arthrospores shed with skin and hair of infected patients are the potential sources of infection for susceptible hosts especially in communal life. There are also growing evidence that arthrospores play vital roles in the recurrence of chronic mycoses.

In view of the importance of arthrospores in the epidemiology of dermatomycoses, our primary effort during the past year was concentrated on the development of physical and chemical means that effectively kills these fungal elements. Prior to this work, there was no published data dealing with the resistance or susceptibility of dermatophytic arthrospores to physical, chemical and biological agents.

We approached this problem systematically and have accumulated sufficient data which, we believe, are useful for developing effective control measures against dermatomycoses both in military and civilian communal life.

The following are the summary of our achievements:

I. Preparation of "pure" arthrospores of dermatophytes.

Arthrospores are, in general, formed within infected tissues by many pathogenic strains of dermatophytes. In many cases, the demonstration of arthrospores within clinical samples is considered to be of diagnostic value. Although the importance of dermatophytic arthrospores in the etiology and pathogenesis of dermatomycoes were recognized by some, it was not possible to test their properties or the susceptibility to various agents because arthrospores were difficult to isolate in pure form and in a large quantity from infected tissues. In an attempt to produce a large quantity of pure arthrospores, we initially tested various physiological and environmental factors to create an in vitro condition similar to the in vivo circumstance under which abundant arthrospores are formed by pathogenic strains of dermatophytes. The oxygen tension, temperature and certain nutritional conditions were found to be most important factors for arthrosporulation in Trichophyton mentagrophytes ATCC 26323, a highly pathogenic strain isolated from a military personnel returning from Vietnam. The results of this preliminary aspect of our project were reported earlier (Emyanitoff, Blumenthal and Hashimoto, Abstract of Annual Meeting of American Society for Microbiology, p. 168, 1977). Based on the results of this study, we subsequently developed a method which can yield a large quantity of pure arthrospores of Trichophyton mentagrophytes in vitro. The outline of the procedure is as follows:

Preparation of arthrospores. Microconidia were produced and purified according to the method described earlier. Approximately 0.15 to 0.2 ml of microconidial suspension (2×10^6 spores/ml distilled water) was inoculated over squares (7 x 7 cm) of sterile cellulose dialysis membrane, which had been placed on Sabouraud dextrose agar (Difco) containing 0.1% sodium acetate. The dialysis membrane was

previously boiled in 1% sodium bicarbonate solution for 10 min, rinsed with distilled water and subsequently sterilized by autoclaving. After incubating the inoculated petri plates at 37°C for 36 h, 4 ml of Sabouraud dextrose broth (Difco) were added aseptically over the dialysis membrane. By this time, hyphae emerging from microconidia adhered to the membrane and the addition of the broth caused no separation of hyphae from the membrane. The plates were placed in a large glass jar (21 cm dia. x 25 cm high) and further incubated under saturated humidity at 37°C for an additional week. By the end of the incubation period, essentially all hyphae were transformed into arthrospores.

The arthrospores were readily removed from the cellulose membrane by gently scraping with a spatula. The harvested arthrospores were routinely filtered through 10 layers of cheesecloth to remove residual hyphae or long chains of arthrospores and subsequently washed in ice-cold distilled water at least 5 times by means of centrifugation (1500 x g, 15 min). After confirming microscopically the absence of hyphal contamination, the arthrospores were dispensed in small vials, tightly sealed and stored at -20°C until use. Under this condition, arthrospores remained fully viable for as long as one year. In most experiments, however, arthropsores were used within 1 to 2 weeks after harvesting.

The availability of a large quantity of pure arthrospores of T. mentagrophytes greatly facilitated the accurate assessment of prospective antidermatomycotic agents on arthrospores (see below). We sent this information to Dr. Michael Lancaster of Letterman Army Institute of Research, Department of Army, where the usefulness of our technique has been confirmed.

II. Effects of physical, chemical and biological agents on T. mentagrophytes arthrospores.

In view of the total lack of information as to the resistance of dermatophytic arthrospores to various physical, chemical and biological agents, we investigated the arthrosporocidal effects of heat, desiccation, ultraviolet light irradiation and various chemicals including glutaraldehyde on T. mentagrophytes arthrospores.

Technical aspects.

Effect of temperature. Arthrospore suspensions (1.5×10^6 cells/ml distilled water) contained in a test tube were heated at 48°, 50°, 55° and 60°C or stored at -20°, 4° and 10°C for various periods of time; then 0.1 ml of heat-treated or chilled spore suspensions were inoculated into 1 ml of Sabouraud dextrose broth and incubated for 15 h at 37°C on a rotary shaker at 300 rev/min. The viability of treated and untreated arthrospores was determined microscopically by counting 200 cells randomly. At the concentration of arthrospores used in the germination system (1.5×10^5 cells/ml), it was usually necessary to examine more than 50 randomly selected fields to count 200 arthrospores. Those arthrospores developing distinctive germ tubes (longer than the spore diameter) were considered viable. By the end of 15 h incubation at 37°C, most germinated arthrospores developed germ tubes as long as 30 to 50 µm.

Effect of ultraviolet light. A 2 ml sample of arthrospore suspension (1.5 x 10^6 spores/ml distilled water) was placed in a small petri dish (40 x 10 mm) and irradiated, with the cover removed, at room temperature for specified time at a distance of 45 cm from the center of a germicidal lamp (Westinghouse Steril lamp 782L-20). Irradiated arthrospores were inoculated into Sabouraud dextrose broth and their viability was determined as described above.

Effect of protein on the survival of desiccated arthrospores. Since our preliminary experiments revealed that T. mentagrophytes arthrospores were highly susceptible to desiccation, the protective effect of selected proteins on the survival of lyophilized arthrospores was investigated. Arthrospores were initially suspended in 5% (w/v) aqueous solutions of powdered skim milk, bovine albumin (crystalline, Sigma Chemical Co.), and gelatin (Difco), and immediately lyophilized. The lyophilized arthrospores were stored at 25°C for specified periods and their viability was determined by the method described above.

Effect of antifungal chemicals. Arthrospores suspended in sterile distilled water were exposed to various concentrations of selected antifungal drugs at 25°C. Nystatin (E. R. Squibb and Sons, Inc.), clotrimazole (Delbay Pharmaceuticals, Inc.), miconazole nitrate (Johnson and Johnson Co.), and griseofulvin (Sigma Chemical Co.) were initially dissolved in dimethyl formamide, appropriately diluted with the solvent and added to spore suspensions so that the final concentration of the solvent would remain 1%. Glutaraldehyde and phenol were obtained from MCB Manufacturing Chemists, and used after appropriate dilution in sterile distilled water. Arthrospores exposed to those chemicals under specified conditions were washed in distilled water 3 times by means of centrifugation (1500 x g, 15 min) and inoculated into Sabouraud dextrose broth for determination of the viability.

RESULTS

Effect of temperature. As shown in Fig. 1, essentially all arthrospores were inactivated within 2 min at 60°C and almost 90% of spores became non viable within 5 min at 50°C. Even at 48°C, approximately 50% of the arthrospore population were killed within 10 min. In contrast to this, arthrospores of T. mentagrophytes were remarkably resistant to chilling and freezing. Arthrospores stored in distilled water at 4°C and 10°C remained viable (90%) for as long as 2 months. More than 95% of the arthrospores could survive for more than 1 year and approximately 65% of the spores remained viable for 2 years when stored in distilled water at -20°C.

Effect of desiccation. T. mentagrophytes arthrospores were exceptionally susceptible to desiccation. Both air dried (25°C) and lyophilized arthrospores lost their viability rapidly during storage, regardless of the storage temperature (Table 1). The extremely high susceptibility of T. mentagrophytes arthrospores to desiccation was reduced significantly when they were dried in the presence of exogenous proteins (Fig. 2).

Effect of ultraviolet light. The kinetics of killing of T. mentagrophytes arthrospores exposed to ultraviolet light from a common laboratory germicidal lamp is illustrated in Fig. 3. Essentially all arthrospores were killed within 10 min under our experimental conditions.

Effect of selected disinfectants and chemotherapeutic agents. Resistance of T. mentagrophytes arthrospores to ethanol, phenol and glutaraldehyde is summarized in Table 2. A remarkably high susceptibility of arthrospores to low concentrations of glutaraldehyde may be worth noting and will be further discussed later. Fig. 4 illustrates the survival of T. mentagrophytes arthrospores exposed to various concentrations of antifungal chemotherapeutic agents. It is evident that arthrospores exposed for 24 h to the drugs at concentrations usually lethal to the hyphal growth could remain viable and were able to germinate once the drugs were removed. Under our experimental conditions, it is not certain whether the drugs adsorbed to arthrospores were completely removed during the washing process. In any event, the high degree of resistance of T. mentagrophytes arthrospores to these chemotherapeutic agents has not been observed previously.

A more detailed account of this aspect of the project will appear in a forthcoming issue of J. Applied and Environmental Microbiology (February, 1978). The manuscript has been accepted for publication.

The observation that T. mentagrophytes arthrospores exposed to several antimycotics at concentrations exceeding the minimal mycelial growth inhibitory level could remain viable (Fig. 4) is believed to have some important clinical and microbiological implications. First, it implies that the administration of these chemotherapeutic agents to dermatomycotic patients whose lesions contain abundant arthrospores may not at all produce anticipated therapeutic effects. This could account for the common experience that excerbation or intermittant recrudescence of infections often takes place in such patients when the administration of the drugs is discontinued. Second, our data suggest that assessment of antidermatophytic activities of prospective drugs to be used in the treatment of ringworm infections should include their effect on arthrospores in addition to vegetative hyphae.

Glutaraldehyde may be an effective antidermatophytic agent since it destroys all known forms of the dermatophyte, microconidia, arthrospores, and vegetative hyphae, at concentrations as low as 0.01%. Further evaluation of glutaraldehyde as an antimycotic and factors affecting its efficacy are now under investigation in our laboratory. (See below).

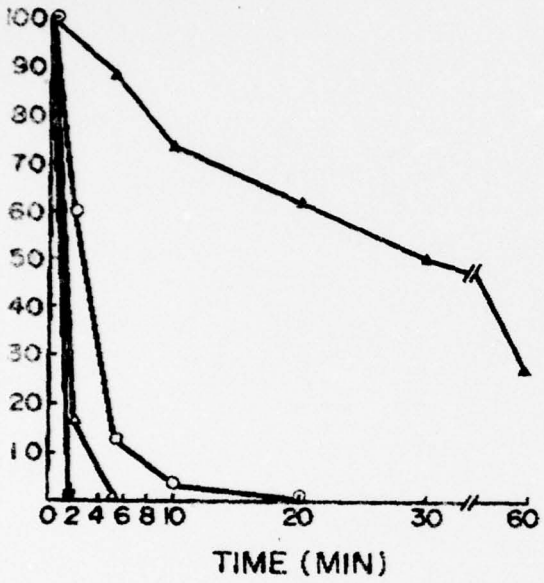
Table 1. Effect of desiccation on the viability of T. mentagrophytes arthrospores

Desiccation conditions	Storage temperature (C)	% survival after 24 h	48 h	148 h
Air dried on cheescloth, 25°C	4°	52	39	0
	25°	34	18	0
	37°	29	11	0
Lyophilized[a]	4°	21	0	0
	25°	11	3	0

[a] Arthrospores suspended in sterile distilled water were lyophilized as described in the Materials and Methods.

Table 2. Arthrosporocidal activities of aqueous ethanol, phenol and glutaraldehyde at 25°C.

Exposure time (h)	% Survival after exposure to Ethanol (70%)	Phenol (0.1%)	Phenol (1.0%)	Glutaraldehyde (0.001%)	Glutaraldehyde (0.01%)	Glutaraldehyde (0.1%)
0.5	0	93	0	97	7	0
1	0	88	0	90	0	0
3	0	85	0	90	0	0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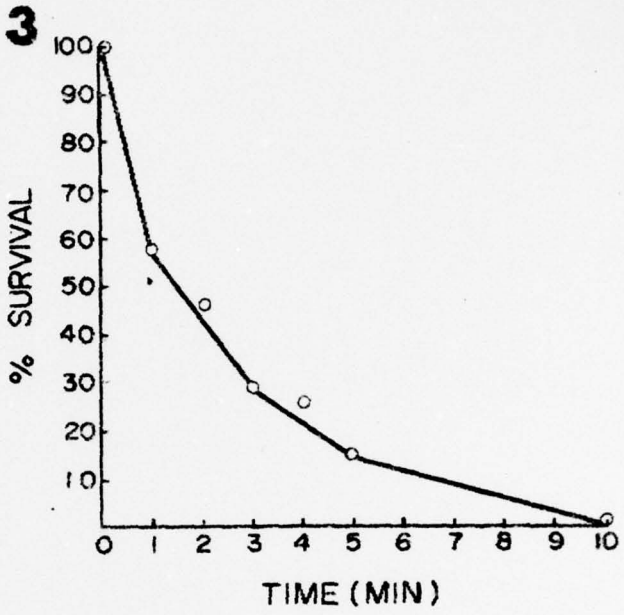
TIME (MIN)

3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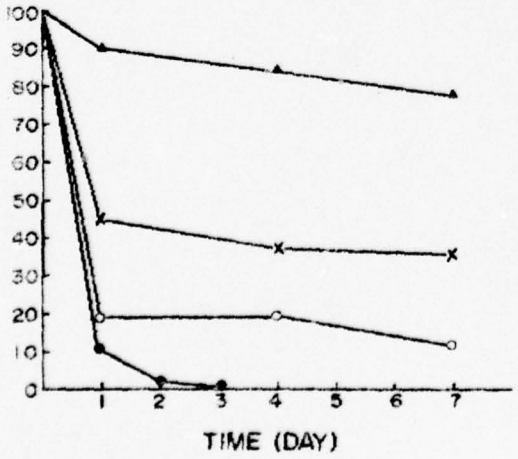
0 1 2 3 4 5 6 7 8 9 10
TIME (MIN)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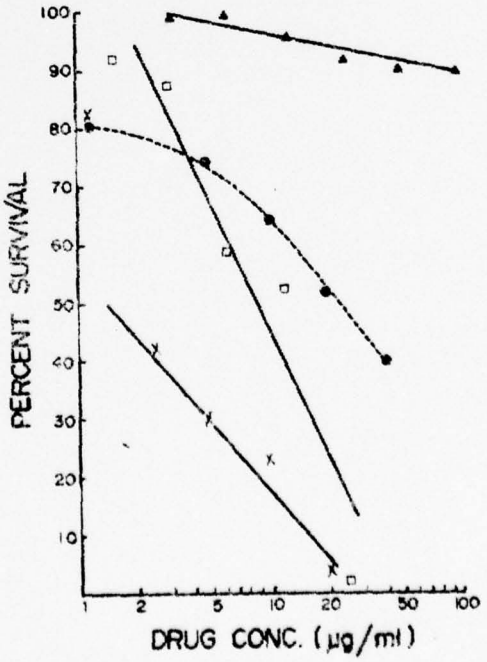
1 2 3 4 5 6 7
TIME (DAY)

4


PERCENT SURVIVAL
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60
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40
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20
10
1 2 5 10 20 50 100
DRUG CONC. (µg/ml)

LEGEND OF FIGURES

Fig. 1. Effect of heat on the viability of T. mentagrophytes arthrospores. Arthrospores were suspended in distilled water and heated for various periods at 60°C (●), 55°C (○), 50°C (■) and 48°C (△) after which the viability was determined as described in Materials and Methods.

Fig. 2: Protective effect of proteins on the survival of lyophilized T. mentagrophytes arthrospores. Arthrospores were lyophilized in the presence of specified proteins as described in Materials, and stored at 25°C. Powdered skim milk (▲), albumin (×), gelatin (□), and control (no proteins, ●). All points except for the day 1 data for gelatin are significantly different ($p < 0.01$) from those controls.

Fig. 3: Effect of ultraviolet light irradiation on the viability of T. mentagrophytes arthrospores.

Fig. 4: Effect of 24 h exposure (25°C) to various concentrations of antidermatomycotic drugs on the survival of T. mentagrophytes arthrospores. Clotrimazole (○), nystatin (×), griseofulvin (△) and miconazole nitrate (□). Arrows in the figure indicate the minimal inhibitory concentration of each drug for hyphal growth.

III. Practical application of glutaraldehyde to disinfection of fabrics contaminated with spores and hyphae of dermatophytes.

The effectiveness of glutaraldehyde as an antidermatophytic agent was tested under simulated laundry conditions. Fabrics contaminated with a predetermined number of T. mentagrophytes spores and hyphae (germinated microconidia) were exposed to detergents or soaps with or without added glutaraldehyde.

Procedure.

A piece of cheese cloth (10 x 10 cm) was sprayed with microconidial or arthrospore suspension and allowed to partially dry. Contaminated clothes were individually soaked in glutaraldehyde solutions (1.0 - 0.001%) at specified temperatures for 30 minutes. The fabrics were removed from the glutaraldehyde solution and washed once in distilled water. After combining the wash solutions for each fabric sample, they were filtered through a Millipore filter (0.45 µm). The spores collected on the filters were allowed to germinate in Sabouraud dextrose broth and the viability of the spores was determined after 6 hours. The results of the experiments are summarized in Tables 3 - 7. It is quite evident that glutaraldehyde at relatively low concentrations killed all forms of T. mentagrophytes very effectively under the simulated washing or laundry conditions.

Table 3. Survival of arthrospores, microconidia and hyphae of T. mentagrophytes in commercially available detergents

SOAP IN WATER (45 C for 15 minutes

	% Viable*		
Recommended conc. of	Arthrospores	Conidia	Hyphae
Axion (enz. mix.)	25-50	50	<10
Borax	50-75	>95	25
Cheer	50-75	>95	25-50
Tide	50-75	>95	<10
Dynamo	50-75	>95	<10
Wisk	50-75	>95	<10
Water only	50-75	>95	25-50
Buffer (pH 7.5)	50-75	>95	25-50

* Determined by microscopical examination as described earlier.

Essentially the same results were obtained when washed at 35 C. When 60 C was employed all forms of T. mentagrophytes were almost completely inactivated within 15 minutes.

Comments:

Dynamo - suds on top
Borax - no suds
Tide - suds throughout
Wisk - few suds
Axion - suds on top
Cheer - suds throughout

Table 4. Survival of arthrospores, microconidia and hyphae of T. mentagrophytes in Axion mixed with glutaraldehyde.

Glutaraldehyde + Axion (45 C for 15 min., 15 min. preincubation)

	Viability (%)		
Recommended conc. of soap plus glutaraldehyde	Arthrospores	Conidia	Hyphae
1.0	0	0	0
0.5	0	0	0
0.1	0	0	0
0.05	0	0	0
0.01	0	0	0
0.005	25	25	<10
0 (Axion only)	25-50	50	<10

Table 5. Survival of arthrospores, microconidia and hyphae of T. mentagrophytes in Tide or Borax containing glutaraldehyde

Glutaraldehyde + Tide* (45 C for 15 min.)

Recommended conc. of soap plus glutaraldehyde	Survival (%) Arthrospores	Conidia	Hyphae
1.0	0	0	0
0.5	0	0	0
0.1	0	0	0
0.05	0	0	0
0.01	25	25	10
0.005	50	50-75	10
0 (Tide only)	50-75	>95	10

Glutaraldehyde + Borax (45 C for 15 min.)

Recommended conc. of soap plus glutaraldehyde	Survival (%) Arthrospores	Conidia	Hyphae
1.0	0	0	0
0.5	0	0	0
0.1	0	0	0
0.05	0	0	0
0.01	10	25	0
0.005	25	25-50	10
0 (Borax only)	50-75	>95	25

Table 6. Survival of arthrospores, microconidia and hyphae of *T. mentagrophytes* in Wisk or Cheer containing glutaraldehyde.

Recommended conc. of soap plus glutaraldehyde	Survival (%) Arthrospores	Conidia	Hyphae
1.0	0	0	0
0.5	0	0	0
0.1	0	0	0
0.05	0	0	0
0.01	0	0	0
0.005	25	50	10-25
0 (Wisk only)	50-75	>95	10

Glutaraldehyde + Cheer (45 C for 15 min.

Recommended conc. of soap plus glutaraldehyde	Survival (%) Arthrospores	Conidia	Hyphae
1.0	0	0	0
0.5	0	0	0
0.1	0	0	0
0.05	0	0	0
0.01	0	0	0
0.005	25-50	50	10
0 (Cheer only)	50-75	>95	25-50

Effect of glutaraldehyde on the survival of dermatophytes parasitizing in isolated human nails.

Dermatomycotic infections involving the nail are most difficult to treat by topical application of conventional antidermatomycotic drugs. To test the feasibility of therapeutic use of glutaraldehyde for the treatment of certain forms of tinea, especially tinea unguinum, we investigated the effect of glutaraldehyde on the survival of T. mentagrophytes parasitizing in isolated human nails. Autoclaved human nail clippings collected from volunteers were infected with T. mentagrophytes in vitro by incubating with germinated (germ tube length approximately 10 microns) microconidia under conditions of high humidity. In a week to 10 days, the fungus not only grew on the surface of the nail but also invaded into the nail. Such infected nails were exposed to various concentrations of glutaraldehyde (pH 7.5) at room temperature (25 C) for 10, 20 and 30 minutes. After exposure, the nails were washed thoroughly in sterile distilled water and then sliced into small pieces (approximately 1 mm^3). Each piece of the nail was placed on Sabouraud's dextrose agar and incubated at 30 C for several days. The results of a typical experiment are summarized in Table 5. It is apparent that 3 to 5% glutaraldehyde completely inactivated T. mentagrophytes parasitizing in the nail within 10 minutes.

Table 7. Lethal effect of glutaraldehyde on T. mentagrophytes parasitizing isolated human nail clippings.

Glutaraldehyde concentration (%)	Growth of T. mentagrophytes on Sabouraud's dextrose agar after exposure for:		
	10 min.	20 min.	30 min.
0	+	+	+
0.01	+	+	+
0.1	+	+	+
1.0	±	-	-
3.0	-	-	-
5.0	-	-	-

\+ Visible growth occurred in 1 week

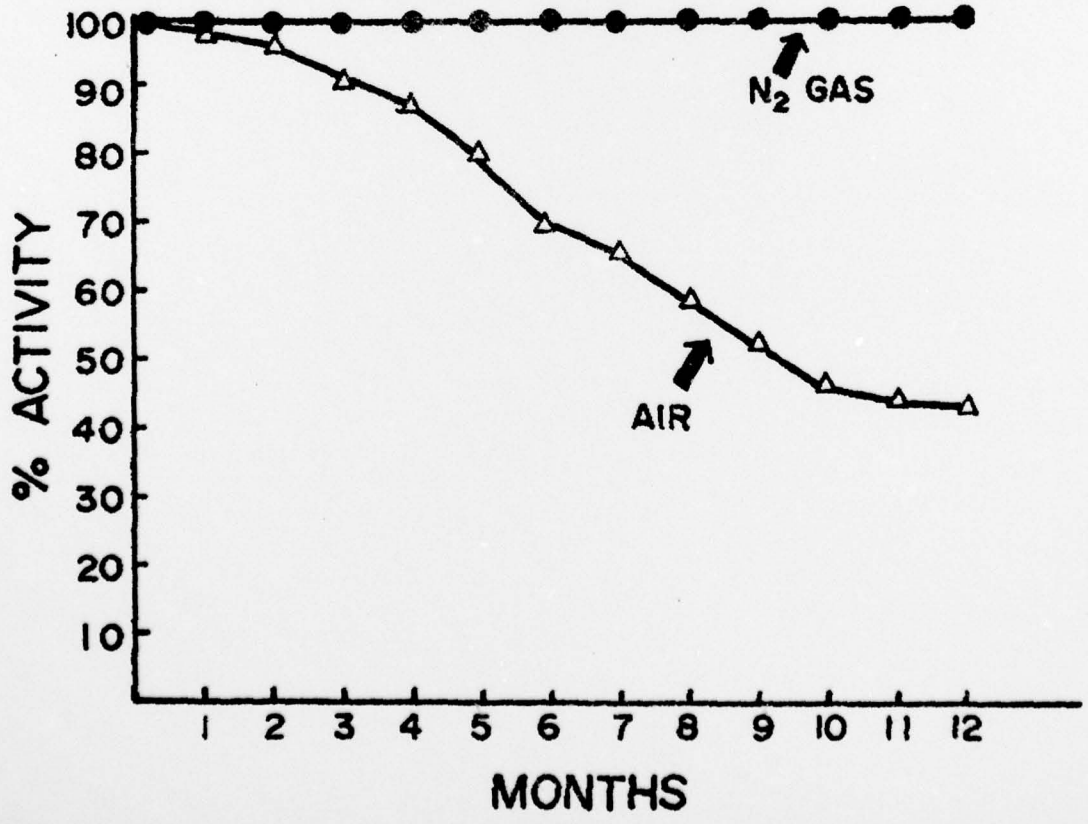
\- No visible growth occurred in 1 week

IV. Stability and preservation of the efficacy of glutaraldehyde as an antidermatophytic agent.

Although glutaraldehyde was previously shown to be a potential and effective antidermatophytic agent, it was noted that it tended to undergo polymerization during storage. In order to find conditions under which the loss of antidermatophytic activity of glutaraldehyde could be kept to a minimum, glutaraldehyde (10% aqueous solution) sealed either in ampoules or plastic bags were stored under various conditions. During storage, the samples were removed periodically and the loss of antidermatophytic activity and the evolution of the polymeric form of glutaraldehyde was quantitatively determined. As reported earlier, the concentrations of monomeric and polymeric forms of glutaraldehyde could be determined spectrophotometrically at 280 and 235 nm, respectively.

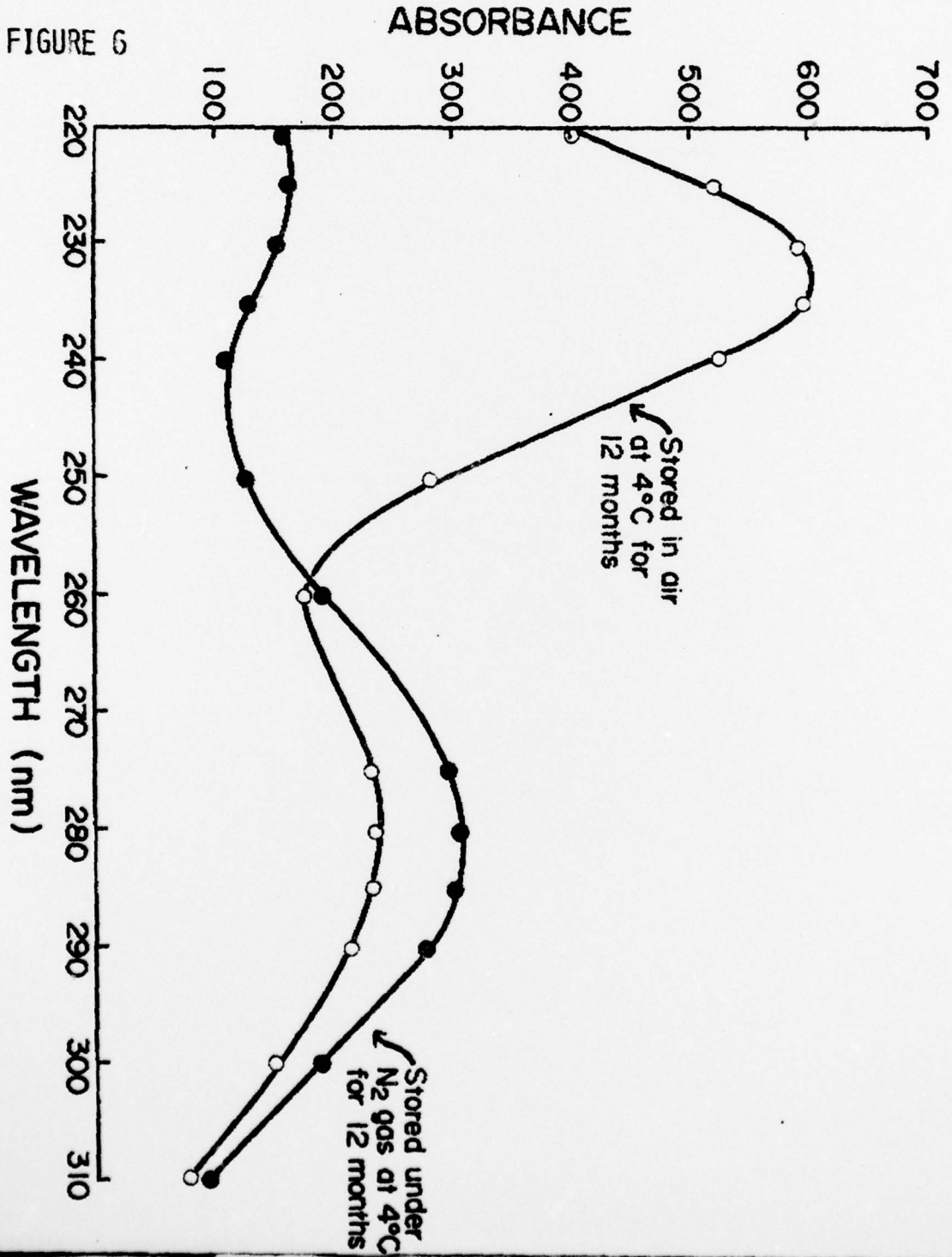
FIGURE 5

EFFECT OF STORAGE CONDITION ON THE SPOROCIDAL ACTIVITY OF GLUTARALDEHYDE



Our investigation revealed also that glutaraldehyde tended to lose its efficacy when stored under aerobic condition even at 0 to 4°C. This loss of antidermatophytic activity was found to be due to formation of dimer or polymer of glutaraldehyde during storage and the degree of the loss of arthrosporocidal activity appeared to coincide with the appearance of an absorption peak at 230-235 nm in UV absorption spectrum (see Fig. 6). Glutaraldehyde stored under *anaerobic* conditions or at below 0°C usually did not produce dimer or polymers. The solution to this question (loss of antifungal activity) was found in our investigation. The ineffective component showing an absorption peak at 235 could be totally and quickly removed by treatment of such glutaraldehyde with activated carbon. We also found that polymerized glutaraldehyde adsorbed to activated carbon could be eluted out by acids (pH 4.0) and its antidermatophytic activity was partially restored upon raising pH to 7.5-8.0.

FIGURE 6



V. Analyses of factors affecting germination of dermatophytic arthrospores.

Since arthrospores of dermatophytes are strongly suspected to play vital roles in recurrence of chronic dermatomycoses and arthrospores shed in skin debris from such patients are a potential source of infections in communal life, we strongly felt that the elucidation of conditions under which these arthrospores initiate their transformation into infective hyphae would deserve critical investigations. Although it is generally assumed that recurrence of chronic dermatomycoses may be related to the immunological deficiency in such patients, our clinical experience of the frequency of cases that are often recalcitrant to therapy can not be explained simply on the basis of immunological deficiency. It is our strong suspicion that arthrospores formed in infected lesions are one of the major factors contributing to such problems. In fact, we recently found that Trichophyton mentagrophytes ATCC 18748 which was used by Jones et. al. in human experiments could not form abundant arthrospores. Infections due to this strain, as shown by Jones et.al. (Arch. Dermatolo. 109: 840-848, 1974) were cured in most cases spontaneously without any recurrence in human volunteers. To understand the role of arthrospores in dermatomycoses, especially its inherent nature of recurrence and resistance to therapeutic agents such as griseofulvin, we investigated the physiological nature of dermatophytic arthrospores, especially their requirements for germination which undoubtedly the first step to recurrence of infections. The results of our study are summarized below. The data in general appear to sufficiently explain why dermatophytic arthrospores can so easily establish a bridgehead in the skin, and also help explain why recurrence or excerbation of chronic dermatomycoses takes place during warm and humid seasons. The data are also useful, in our opinion, to select derivatives of certain germination inducers for possible use in the treatment of dermatomycoses. The analyses of the data also raise a serious doubt as to the validity of conventional methods of treatments; continuous administration of oral or topical antidermatophytic agents without paying enough attention to the susceptibility of arthrospores to these drugs. It is also noteworthy that most of antidermatomycotic agents have been tested, prior to FDA approval, only for their effects on hyphal or vegetative forms but not on arthrospores which are often present in the infected loci.

TECHNICAL:

Germination system. The germination system used in the present investigation was essentially the same as described earlier for germination of microconidia. Test tubes, 22 by 75 mm, containing 0.5 ml of T. mentagrophytes arthrospore suspension were incubated in the presence of an appropriate concentration of a prospective germination agent on a rotary shaker (model G10, New Brunswick Scientific Co., New Brunswick, N.J.) at 200 rpm under specified conditions. Unless otherwise stated, the germination system contained 0.8×10^5 spores per tube. Samples were removed by appropriate intervals, and percentages of arthrospores developing visible germ tubes were estimated microscopically by counting a total of 100 arthrospores. When multiple samples were removed at one time, glutaraldehyde (25%, MCB Manufacturing Chemists) was added to each tube to a final concentration of 5%, and percentages of germination were determined later as described above.

Effects of temperature and pH on germination. The effect of temperature on arthrospore germination was tested by incubating test tubes containing the mixture of arthrospores and germination inducers in a water bath shaker (New Brunswick model R76) or in a refrigerated water bath (Haake model FK2) preadjusted to a desired temperature. The effect of pH on germination was studied by inoculating spores into germination media preadjusted to desired pH by 0.1 N NaOH or HCl.

Effects of NaCl and antifungal chemicals on germination. Sodium chloride and other antifungal chemicals were incorporated in the germination media at desired concentrations. Those chemicals insoluble in water were initially dissolved in dimethyl formamide and subsequently diluted 100 times with the germination media to obtain desired concentrations.

Effect of oxygen on germination. The requirement of oxygen for the initiation of germination was tested by incubating test tubes containing the mixture of arthrospores and germination inducers in a GasPak jar (BBL, Cockeysville, Md.) at 37°C for 24 h.

Determination of the minimal inhibitory concentration for the hyphal growth of T. mentagrophytes. To compare the effect of antifungal chemicals on germination and postgerminative growth, the minimal inhibitory concentration of each drug for the hyphal growth of T. mentagrophytes was determined. A series of test tubes, 22 by 77 mm, containing 0.5 ml of Sabouraud dextrose broth and various concentrations of drugs as obtained by the twofold dilution method, were inoculated with approximately 10^5 germinated microconidia and incubated, without agitation, at 30°C for 1 week. Germinated microconidia were obtained as described earlier. The minimal concentration of a drug contained in the tube showing neither surface nor submerged growth was macroscopically determined and was taken as the minimal inhibitory concentration for the hyphal growth.

Phase-contrast photomicroscopy. The microscopic appearance of the wet-mouthed spores was examined with a phase-contrast microscope by using an oil immersion objective (dark medium, x 100; numerical aperture, 1.25; Nikon). Photomicroscopy was made on panchromatic film (Kodak Plus-X) with a Nikon camera equipped with an automatic exposure system attached to a phase-contrast microscope.

Chemicals. All of the amino acids and peptides used were chromatographically pure and were purchased from Sigma Chemical Co., St. Louis, Mo. Unless specifically mentioned otherwise, all amino acids and dipeptides refer to the L-isomers. Carbohydrates and other biochemicals were from Nutritional Biochemical Co., Cleveland, Ohio. Sabouraud dextrose broth, neopeptone, and vitamin-free Casamino Acids were purchased from Difco. All other chemicals were of reagent grade. Amphotericin B and nystatin were kindly supplied by E. R. Squibb and Sons, Princeton, N.J., clotrimazole by Delbay Pharmaceuticals, Inc., Bloomfield, N.J. and tolnaftate by Schering Laboratories, Kenilworth, J.J. Griseofulvin was purchased from Sigma Chemical Co.

RESULTS

Germination of dormant arthrospores. Both freshly harvested arthrospores and frozen stocks of arthrospores of T. mentagrophytes (referred to as dormant arthrospores in the subsequent discussion) germinated fairly rapidly, i.e., in less than 7 h, when incubated in rich media such as Sabouraud dextrose broth or in 1% vitamin free casamino acids (Fig. 7A). However, dormant arthrospores germinated very poorly or not at all (<10%).in sodium or potassium buffer (0.05 M, pH 6.5) containing single amino acids or peptides (Table 8 and Fig. 7A). Essentially no germination (<10%) of dormant arthrospores took place during incubation at 37°C for 15 h in distilled water, tap water, physiological saline solution (0.85% NaCl), sodium or potassium phosphate buffer (0.05 M, pH 6.5) or in a basic salt solution of Merz et al. containing 2 mM $MgSO_4$, 0.7 μM $FeCl_3$ and 0.6 μM $MnSO_4$.

Germination of activated arthrospores. When dormant arthrospores were stored in distilled water at 25°C for 24 h, (these arthrospores will be referred to as activated arthrospores in subsequent discussion) they became conditioned to germinate either spontaneously in distilled water, phosphate buffer and basic salt solution, or more effectively in the presence of certain single amino acids and peptides (Fig. 7B and Table 8). Treatment with sublethal doses of heat also appeared to facilitate the activation of dormant arthrospores (Table 9). Activated arthrospores germinating spontaneously in distilled water, buffer or saline solution or in the presence of single amino acids, peptides or sugars could develop only short germ tubes (up to several micra). Further elongation of germ tubes generally required the supplementation of additional nitrogen and carbon sources.

Effect of chemicals on arthrospore germination. The following individual carbohydrates, organic acids, purines, pyrimidines and nucleosides failed to induce germination of either dormant or activated T. mentagrophytes arthrospores.

Carbohydrates and organic acids. Carbohydrates and organic acids (0.5% in sodium phosphate buffer, 0.05 M, pH 6.5) were as follows: D-glucose, D-mannose, L-mannose, D-fructose, D-galactose, D-ribose, L-arabinose, D-xylose, L-xylose, D-rhamnose, D-turanose, D-cellobiose, D-melezitose, D-trehalose, D-melibiose, sucrose, L-sucrose, D-sorbitol, L-arabitol, iso-erythritol, dulcitol (galactitol), glycerol, adonitol (ribitol), arabic acid, inulin, salicin, pyruvate (na), succinate (Na), α-ketoglutarate (Na), N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, and D-galactos-amine hydrochloride.

Purines, pyrimidines, and nucleosides. Purines, pyrimidines, and nucleosides (20 mM in 0.1 M sodium phosphate buffer, pH 6.0) were as follows: adenine, cytidine sulfate, guanine, uracil, xanthine, hypoxanthine, adenosine, cytosine, guanosine, uridine, 5-methylcytosine-hydrochloride, and inosine.

The effect of concentration of vitamin free casamino acids on germination of activated arthrospores of T. mentagrophytes is shown in Table 10. It is evident that T. mentagrophytes arthrospores were able to germinate at concentrations as low as 0.001%. They were able to initiate germination in the presence of single amino acids such as L-alanine, glycine, L-leucine, L-serine, and L-threonine at concentrations as low as 0.02 mM. D-isomers of certain amino acids could also initiate germination but, in general, less effectivity than L-isomers (Table 11). D-isomers of the other amino acids listed in Table 1 were either inhibitory to germination or caused germination no more than the controls (15%).

Effect of temperature and pH on arthrospore germination.- Germination of T. mentagrophytes arthrospores could take place in Sabouraud dextrose broth under wide ranges of temperature (Fig. 8) and pH (Fig. 9). However, the optimal temperature for germination un vitamin-free casamino acids or in L-leucine was found to be 37°C. Similarly, the optimal pH for germination in vitamin-free casamino acid or in L-leucine was 6.5.

Effect of spore concentration on arthrospore germination. Apparently, the concentration of arthrospores critically affected the rate of germination induced by amino acids. Essentially no germination took place, even under optimal conditions, when the concentration of arthrospores reached 10^7 cells per ml (Table 12). When properly diluted, all these arthrospores underwent germination normally.

Requirement of oxygen for arthrospore germination. Oxygen appeared to be essential for the initiation of germination of arthrospores. No germination occurred when activated arthrospores were incubated in Sabouraud dextrose broth or in 1% vitamin-free casamino acids under strict anaerobic conditions.

Effect of NaCl and certain antidermatophytic compounds on arthrospore germination. As shown in Table 13, germination of T. mentagrophytes arthrospores in vitamin free casamino acids was only slightly inhibited by high concentrations of NaCl (5%). The effects of various antifungal chemicals on germination of T. mentagrophytes arthrospores were shown in Table 14. Apparently the germination process of T. mentagrophytes arthrospores was less susceptible to certain antifungal agents as compared with the growth of the hyphal form.

TABLE 8. *Germination of dormant and activated arthrospores of T. mentagrophytes induced by L-amino acids and peptides*[a]

Compound	% Germination[b]	
	Dormant	Activated
Amino acids		
Alanine	<10	85
Arginine hydrochloride	<10	53
Asparagine	<10	23
Aspartic acid	<10	31
Cysteine	<10	25
Cystine	<10	18
Glutamine	<10	30
Glutamic acid	<10	18
Histidine hydrochloride	<10	38
Hydroxyproline	<10	20
Isoleucine	<10	62
Leucine	<10	80
Lysine hydrochloride	<10	14
Methionine	<10	30
Phenylalanine	<10	58
Proline	<10	46
Serine	<10	69
Threonine	<10	68
Tryptophan	<10	52
Tyrosine	<10	71
Valine	<10	68
Glycine	<10	74
Leucine analogs		
Leucyl methyl ester	<10	37
Leucyl ethyl ester	<10	19
N-carbonyl-leucine	<10	21
Peptides		
Leucyl-leucine	<10	57
Leucyl-valine	<10	72
Leucyl-tyrosine	<10	50
Valyl-leucine	<10	13
Valyl-tyrosine	<10	37
Valyl-valine	<10	29
Leucyl-leucyl-leucine	<10	31
Tetra-alanine	<10	61
Penta-alanine	<10	41
Control		
Distilled water	<10	14
Sodium phosphate buffer (0.05 M, pH 6.5)	<10	22
Vitamin-free Casamino Acids	>95	>95

[a] Activation of dormant *T. mentagrophytes* arthrospores was achieved by incubation in sterile distilled water at 25°C for 24 h.

[b] Percentage of germinated arthrospores after incubation of arthrospores at 37°C for 15 h in the presence of 10 mM of each compound in sodium phosphate buffer (0.05 M, pH 6.5), determined as described in the text.

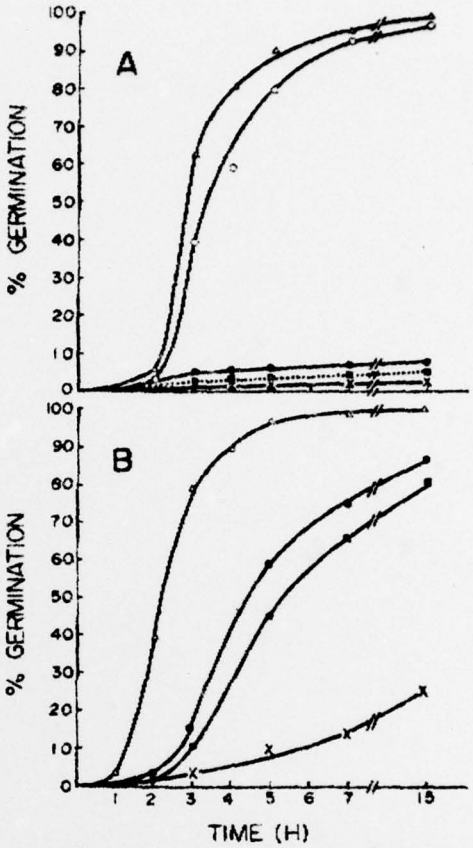


FIG. 7. *Kinetics of germination of dormant (A) and activated (B) arthrospores of T. mentagrophytes in Sabouraud dextrose broth (△), 1% vitamin-free Casamino Acids (○), 10 mM L-leucine (■), 10 mM L-alanine (●), and 0.05 M sodium phosphate buffer, pH 6.5 (×).*

TABLE 9. *Effect of storage temperature upon subsequent germination of dormant T. mentagrophytes arthrospores*[a]

Treatment	VFCA[b]	% Germination in:				
		L-Alanine	L-Leucine	L-Serine	Glycine	Control[c]
None	>95	<10	<10	<10	<10	<10
4°C, 24 h	>95	<10	<10	<10	<10	<10
25°C, 24 h	>95	85	80	69	74	24
45°C, 10 min	>95	48	60	53	45	18
45°C, 20 min	76	79	71	42	52	16
50°C, 15 min	24	<10	<10	<10	<10	<10

[a] Dormant arthrospores suspended in distilled water were subjected to temperature treatment as specified and subsequently allowed to germinate at 37°C for 15 h in sodium phosphate buffer (0.05 M, pH 6.5) containing 1% vitamin-free Casamino Acids or 10 mM amino acids.

[b] 1% vitamin-free Casamino Acids.

[c] Sodium phosphate buffer (0.05 M, pH 6.5).

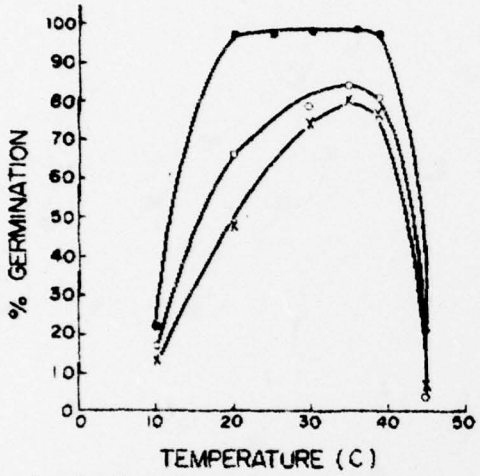


FIG. 8. *Effect of temperature on germination of activated T. mentagrophytes arthrospores. Arthrospores were germinated for 15 h in Sabouraud dextrose broth (●), 1% vitamin-free Casamino Acids (○), and 10 mM L-leucine (×). pH of the media was adjusted to 6.5.*

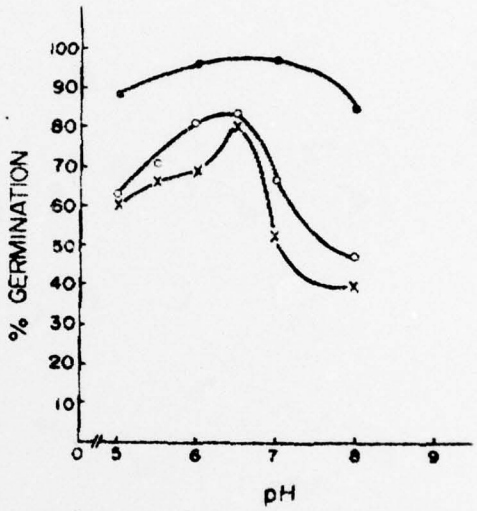


FIG. 9. *Effect of pH on germination of activated T. mentagrophytes arthrospores. Arthrospores were germinated for 15 h in Sabouraud dextrose broth (●), 1% vitamin-free Casamino Acids (○), or 10 mM L-leucine (×), adjusted to the indicated pH.*

TABLE 10. *Effect of concentration of vitamin-free Casamino Acids (VFCA) on germination of T. mentagrophytes arthrospores*

Concn of VFCA (%)	% Germination[a]
1.0	>95
0.5	>95
0.1	93
0.05	89
0.01	76
0.005	76
0.001	73
0.0	21

[a] Percentage of germinated arthrospores after incubation at 37°C for 15 h in the specified concentration of VFCA in sodium phosphate buffer (0.05 M, pH 6.5), determined as described in the text.

TABLE 11. *Germination of activated arthrospores of T. mentagrophytes induced by D-amino acids*

D-Amino acids	% Germination[a]
Alanine	26
Asparagine	28
Isoleucine	31
Leucine	33
Serine	31
Threonine	32
Sodium phosphate buffer (0.05 M, pH 6.5)	15
Distilled water	15
1% vitamin-free Casamino Acids	>95

[a] Percentage of germinated arthrospores after incubation at 37°C for 15 h in the presence of 10 mM of each compound in sodium phosphate buffer (0.05 M, pH 6.5), determined as described in the text.

TABLE 12. *Effect of cell concentration on germination of activated T. mentagrophytes arthrospores*

Spore concn cells/ml	% Germination[a]		
	VFCA[b]	L-Alanine	L-Leucine
0.8×10^6	>95	85	80
3.4×10^5	81	66	64
8.5×10^5	84	8	31
1.7×10^5	39	3	5
3.4×10^5	23	1	1
1.0×10^5	0	0	0

[a] Arthrospores were activated by incubating in sterile distilled water at 25°C for 24 h and were then germinated at 37°C for 15 h in sodium phosphate buffer (0.05 M, pH 6.5) containing 1% vitamin-free Casamino Acids or 10 mM amino acids.
[b] 1% vitamin-free Casamino Acids.

TABLE 13. *Effect of NaCl on germination of T. mentagrophytes arthrospores*

Concn of NaCl (%)	% Germination[a]
0	>95
0.5	>95
1.0	>95
2.0	>95
5.0	58
10.0	9

[a] Percentage of germinated arthrospores after incubation at 37°C for 15 h in the presence of 1% vitamin-free Casamino Acids supplemented with the specified concentration of NaCl in sodium phosphate buffer (0.05 M, pH 6.5), determined as described in the text.

TABLE 14. *Effects of antifungal compounds on germination of T. mentagrophytes arthrospores*

Concn of compound[a] (μg/ml)	% Germination[b]					
	Amphotericin B (5 μg/ml)	Clotrimazole (0.5 μg/ml)	Cycloheximide (100 μg/ml)	Griseofulvin (12.5 μg/ml)	Nystatin (5 μg/ml)	Tolnaftate (12.5 μg/ml)
Control	>95	>95	>95	>95	>95	>95
0.01	>95	>95	>95	>95	>95	>95
0.1	>95	>95	>95	>95	90	>95
0.5	10	>95	>95	>95	88	>95
1.0	2	>95	>95	>95	47	>95
5.0	0	85	>95	>95	8	>95
10	0	72	>95	>95	8	>95
25	0	56	>95	>95	6	86

[a] These compounds are initially dissolved in dimethyl formamide and subsequently diluted 100 times to specified concentrations with 1% vitamin-free Casamino Acids (VFCA). Control contained 1% dimethyl formamide in 1% VFCA.
[b] Percentage of germinated arthrospores after incubation at 37°C for 15 h in the presence of 1% VFCA supplemented with specified final concentrations of antifungal compounds in sodium phosphate buffer (0.05 M, pH 6.5), determined as described in the text. Figures in the parentheses represent the minimal inhibitory concentrations for hyphal growth of *T. mentagrophytes* as determined by the method described in the text.